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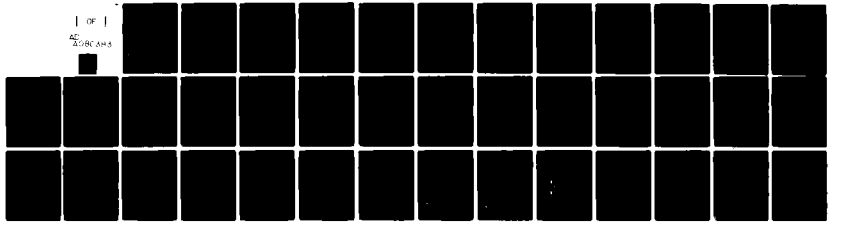

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(6) PREPARATION OF A UNIVERSAL BLOOD DONOR TYPE.

by

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and

Biological Chemistry

Ann Arbor, Michigan 48109

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The A-zyme from Cl perfringens has been purified over 8,000 fold with a yield of 17%. The contaminating enzymes: sialidase, β-galactosidase and β-N-acetylglucosaminidase, that have persistently co-purified with it, have now been reduced to 1 - 2% of the level of A-zyme activity. Treatment of

beta

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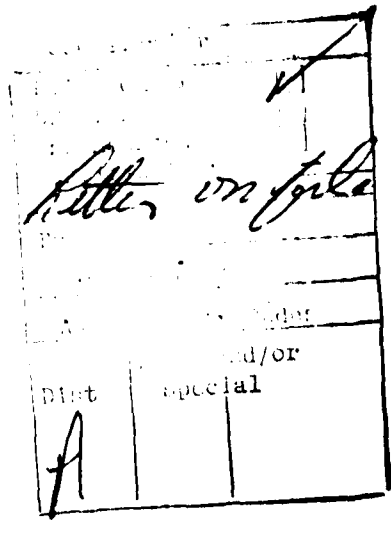
various mucins with the purified enzyme released predominantly N-acetylgalactosamine from A-active glycoproteins, and only trace amounts of N-acetylglucosamine. This action is accompanied by a loss of A activity and the development of (o)H cross-reactivity. SDS-PAGE analysis results in the detection of two bands of activity when tested with p-nitrophenyl-α-N-acetylgalactosaminide, with R_e=0.3 and 0.44, in the relative amounts of 1:2. However, only the 0.44 band shows enzymatic activity with A^+ RBC, glycoproteins and terminal non-reducing N-acetylgalactosamine containing oligosaccharides derived therefrom.

The B-zyme has now been purified 2,500 fold from Cl. sporogenes, Maebashi, with a recovery of 4%. No other glycosidase was detected as a contaminant. Disc gel electrophoresis demonstrated the presence of several protein staining bands, with the α-galactosidase activity restricted to essentially one band at R_e=0.28. The enzyme has no action on Gal α1→2 Glc, Gal α1→3 Gal, or Gal α1→6 Gal α1→6 Glc.



ANNUAL REPORT #4

DECEMBER 1, 1978 - NOVEMBER 30, 1979

TABLE OF CONTENTS

DETAILED REPORT DECEMBER 1, 1978, NOVEMBER 30, 1979

A. PERSONNEL

Dr. Tadanisa Kogure has returned to Maebashi, Japan, after a two year sojourn in our laboratories. He brought the organism Cl. sporogenes (Maebashi) with him, and initiated the work on the purification of B-zyme (α1→3-D-galactosidase). The work is now being completed by Mr. George N. Lowrie.

Dr. Gerald N. Levy has left our group to join another department here on campus. He was responsible for the purification of the A-zyme from Cl. perfringens. The position he vacated is now open, and it is hoped to fill it with an immunologically oriented biochemist, as will be elaborated upon below.

Dr. Bard Smedsrod has recently joined us from Tromso, Norway. His Ph.D. thesis was on work done with macrophages and liver Kupffer cells, an area eminently suitable to the project at this juncture. He is a recent arrival to the team and already has made a positive impact on the progress of the project.

B. A-ZYME FROM CLOSTRIDIUM PERFRINGENS

1. A-zyme$^+$ and A-zyme$^-$ Strains. In a previous report, (1), we had described our frustrations at the repeated disappearance and reappearance of the A-zyme activity in our stocks of Cl. perfringens.

Discussions with a number of colleagues on campus regarding the possible cause of this erratic behavior resulted in a collaborative effort with Dr. R. Olsen, Dept. of Microbiology.

The principal cause identified was a contamination of our stocks of Cl. perfringens with an organism that does not produce the enzyme. More careful attention to sterile techniques, use of Cl. perfringens stored a) frozen in glycerol or, b) freeze dried, has so far obviated the difficulty.

2. Purification of the A-zyme.

The current purification procedure finally adopted is outlined in Table I, which indicates that the A-zyme has been purified a) some 8,000 fold with respect to total protein and, b) 4,500 fold with respect to sialidase, a serious contaminant in view of its effect on the viability of erythrocytes (2), and c) 900 - 2,000 fold with respect to β-galactosidase and β-N-acetylglucosaminidase, respectively.

3. Purity of A-zyme

The final product from the purification shows no reactivity with the artificial p-nitro-phenyl glycoside substrates with the exception of the p-nitro-phenyl derivative of galactose and N-acetylglucosamine (Table II), as has already been alluded to.

Examination of the sugars released, after prolonged incubations, from a number of blood group active glycoproteins, indicated that only N-acetyl-galactosamine was released, and this only from A+ glycoproteins, with trace amounts of N-acetylglucosamine from hog gastric mucin.

Protease activity was assayed with ^{14}C-methemoglobin. No release of radioactivity was observed with the purest preparation of the enzyme although there was significant amount of protease activity in the initial culture fluid.

Polyacrylamide gel-electrophoresis of A-zyme from step 6 was performed in 4% and 5.5% acrylamide gels. Gels were stained with Coomassie blue or sliced into 1 mm slices and incubated with p-nitrophenyl-α-N-acetylgalactos-aminide to locate the position of the enzyme. Six protein bands were detected, with the enzymatic activity corresponding to R_e=0.39 (Fig 1) in the 4% gels and R_e=0.24 in the 5.5% gels.

4. Properties of the A-zyme

The A-zyme activity is stable to freezing at all stages of purification, however, reducing agents such as DTT should be included in all buffers used for storing the enzyme. Purified A-zyme can be lyophilized without appreciable loss of activity. The enzyme may be precipitated by dialysis against pH 4.5 acetate buffer containing DTT. The redissolved enzyme will have 95 to 100% of the starting activity.

The A-zyme shows optimal activity at pH5, with approximately 90% of peak activity remaining at pH 7.0, with either the artificial or blood group A^+ active substrates. The effect of ions on the activity of the enzyme is shown in Table III.

5. Specificity

The results shown in Table IV indicate that GalNAc is the only product detected upon incubation of purified A-zyme with A^+PSM, but that incubation did not give information as to how much of the total acetylhexosamine present in the mucin could be released by A-zyme. In order to make such a determination and to ascertain the bonds susceptible to hydrolysis by A-zyme, a known amount of mucin was incubated and the amount of GalNAc obtained from it determined. A sample of A^+PSM was assayed for total hexosamine and the distribution of various oligosaccharide chains determined (3). Table V shows that of the 434 μmole of oligosaccharide chains found per gm of A^+PSM, only oligosaccharides A_5, N_4 and N, can release free GalNAc from the non-reducing end of the chains. These represent 23+96+71 residues respectively totaling 190 GalNAc residues out of a total of 553 GalNAc residues to be found in the mucin, namely $\frac{190}{553}$=34.4%. Incubation of A^+PSM (330 nmole of total hexosamine) with A-zyme resulted in the release of 110 nmoles of hexosamine at the end of two hours, Fig 2. This represents 33% of the total hexosamine which is very close to that anticipated from the above calculations.

In order to determine if the lack of further GalNAc release is due to decay of the A-zyme or exhaustion of available substrate, the experiment illustrated in Fig 2 was performed. Addition of fresh enzyme at the 4 hr time point had very little effect, releasing only a few additional nmoles of GalNAc. Addition of $A^{+}PSM$, equal in amount to the starting material, had the dramatic effect of releasing an additional 32% of total GalNAc present in the substrate. The quantity of GalNAc released is the amount to be expected from hydrolysis of the non-reducing GalNAc residues from oligosaccharides N_4 and A_5 and the GalNAc of N_1.

GalNAc was the only product found when the incubation mixture was examined by chromatography and electrophoresis.

Polyacrylamide gel electrophoresis in SDS was found to be useful for both purification and for determining the specificity of A-zyme. The glycosidase activities in the A-zyme preparation were found to be stable to treatment with 2% (w/v) SDS at 37°. An aliquot of enzyme treated with SDS retained 90-100% of its A-zyme activity and at least 70% of the activity of the contaminant glycosidases. After treatment with 2% SDS for 60 min, A-zyme was run in 5.5% acrylamide gels with 0.1% SDS in the gel and buffer solutions. Gels were stained for protein with Coomassie Blue or examined for activity by slicing and incubation with various substrates. Figures 3 A-D show the results of this experiment. Three major protein bands were found along with a few minor bands. Two of the major proteins coincide with α-N-acetylgalactosaminidase activity at R_e=0.28 and R_e=0.40. The finding of two distinct peaks reacting with p-nitrophenyl-α-acetylgalactosaminide is noteworthy. Additionally, the contaminant activities of β-galactosidase and β-N-acetylglucosaminidase are separated from both of the "A-zyme" peaks, at R_e=0.47 and 0.60 respectively.

When SDS gels were sliced and incubated with various mucins, GalNAc was released from A^+PSM as indicated in Fig 3B. The substrate specificity of the two isozymes is given in Table VI.

6. Enzyme Kinetics with the Various Substrates

The rate of release of GalNAc by A-zyme from various substances was determined for several substrate concentrations. The results were plotted as double reciprocals, and K_M and V_{Max} values obtained. The data, presented in Table VII, indicate that there is only a minor difference in K_M values for the neutral tetrasaccharide alditol and the acidic pentasaccharide alditol from A^+PSM. This suggests that the presence of sialic acid near the reducing end of the carbohydrate chain does not affect the binding and action of A-zyme at the non-reducing end. This may be an important consideration in a study of the action of A-zyme on RBC where sialic acid residues abound. It can also be seen from Table VII that, as expected, the reaction is most rapid with low molecular weight substrates and slower with the high molecular weight mucins.

7. Serological Changes Resulting from the Action of A-zyme

Enzyme from step 6 (1.5 unit) was incubated with 200 µl packed human erythrocytes in 500 µl PBS for 30 min at 37°. Control incubations omitted the enzyme. After washing and diluting the erythrocytes, hemagglutination titers were determined using micro-titer techniques. Human anti-A and anti-B typing sera were used as were lima bean lectin as an anti-A reagent and *Lotus tetragonolobulus* lectin as anti-H. Table VIII shows that under the conditions used, the A-zyme preparation can significantly reduce the A activity of human erythrocytes producing a simultaneous appearance of H activity (the O blood group). Furthermore, the enzyme did not alter the blood group activity or titers of human O or B erythrocytes.

C. B-ZYME FROM CLOSTRIDIUM SPOROGENES (MAEBASHI)

1. Purification of the B-zyme

As described in Report #3, the preparation of the B-zyme from Cl. sporogenes resulted in a 40 fold purification from the starting bacterial culture filtrates with an overall yield of 57.6%. With the change in personnel several improvements were adopted with an overall purification and yield of enzyme as shown in Table IX.

2. Purity of B-zyme

As previously reported (Report #3) the preparation of the B-zyme was free of sialidase, fucosidase and other glycosidases that appear to contaminate the crude A-zyme from Cl. perfringens.

Since we now report a 2518 fold purer preparation of the B-zyme, we reexamined the present preparation of B-zyme in PAGE (at pH 8.5, and 6.5% gel with a 3% spacer gel). Figure 4 shows the pattern of coomassie staining protein components and the position of the B-zyme at R_c=0.28 as determined by the activity of the slices. The elution of the enzyme from the gels is good (76%) and the eluted enzyme will be rerun at pH 6.5 and in 5.5% and 7.5% polyacrylamide gels and SDS-PAGE to establish its purity.

3. Properties of the B-zyme

The purified enzyme appears to be stable for three months stored at 4°C and retained 75% of activity after freeze drying. It lost all its activity after 10 min at 56°C.

The enzyme was found to have a broad range of activity between pH 5.5 to 7.0, and the enzyme appeared to be stable for 20 hrs. at 4° in buffers ranging from pH 5.0-9.0. The enzyme loses activity below pH 5.0.

4. Substrate Specificity

The enzyme has no action on the artificial substrate p-nitrophenyl-α-galactoside. Moreover, it released no detectable galactose from the

following oligosaccharides Gal $1\overset{\alpha}{\rightarrow}2$ Glucose, Gal $1\overset{\alpha}{\rightarrow}3$ Gal or from Gal $1\overset{\alpha}{\rightarrow}6$ Gal $1\overset{\alpha}{\rightarrow}6$ Glucose (see Fig 5) under conditions which rapidly released galactose from the ovarian B cyst alditol substrate. It was surprising to us that the disaccharide Gal $1\overset{\alpha}{\rightarrow}3$ Gal, carrying the B determinant did not react. This was obtained from Dr. E. A. Kabat, and there is no doubt as to its authenticity. It would imply that the presence of fucose, as shown below, is important to the specificity of the enzyme,

$$\text{Gal } 1\overset{\alpha}{\rightarrow}3 \text{ Gal}\rightarrow \underset{\uparrow\alpha(1,2)}{\text{Fuc}} \quad \xrightarrow{\alpha\text{Galase}} \quad \text{Gal}\rightarrow \underset{\uparrow\alpha(1,2)}{\text{Fuc}}$$

B (O)H

Tests are underway to confirm this rather unexpected finding.

D. BIOLOGICAL PROPERTIES OF ENZYME-TREATED ERYTHROCYTES

1. Viability Studies:

We have previously demonstrated that sialidase treated erythrocytes very rapidly lose their viability in circulation (2). Subsequent experiments demonstrated that the asialo-erythrocytes readily gave rosettes with Kupffer and spleen monocytes (4). Initial indications were that peritoneal macrophages also give rosettes (5).

It was decided to concentrate on developing an assay with the peritoneal macrophages since they are more easily isolated and cultured than are the Kupffer cells. Attempts to make the rosette assay quantitative were not very successful.

In the belief that the peritoneal cavity would be a more physiological environment to study rosette formation and ultimately erythrophagocytosis, we injected ^{51}Cr-labeled sialidase-treated, asialo erythrocytes (aRBC) and untreated, control, erythrocytes (cRBC) into two separate rats. In both cases, the erythrocytes left the peritoneal cavity. An overall body

survey of the sacrificed rats, Table X, indicated that the bulk of cRBC were returned to the circulation, while the aRBC were sequestered in the liver. In both experiments homologous rat erythrocytes were used.

2. Route of Injection

A comparison was made of the three alternative routes of transfusion of the RBC; intravenous, (i.v.), intraperitoneal, (i.p.), and intracutaneous, (i.c.). Table XI shows the results obtained and indicates that the i.c. route causes hemolysis and is undesirable. While the intravenous route appears to give the same overall response as does the intraperitoneal route, the latter is preferred for two reasons; - a) it is operationally far simpler than i.v., when using small animals like a rat, with the assurance that all the radioactively labeled erythrocytes are effectively injected into the animal, and b) the aRBC are sequestered directly and completely in the liver, as contrasted with intravenous where some 10% of the labeled RBC are taken up in the spleen.

3. Distribution of cRBC and aRBC within the Various Compartments

The distribution of cRBC and aRBC in the various compartments of blood, liver and peritoneal cavity are shown in Table XII. The results indicate that cRBC simply return to the RBC compartment of blood while aRBC are concentrated mainly in the non-parenchymal cells of the liver, very little, if any is taken up by the peritoneal macrophages or blood monocytes.

4. Time Curve for Attainment of Equilibrium with cRBC and aRBC

The distribution of ^{51}Cr labeled cRBC and aRBC was followed over a period of 22 hrs in rats, with the results as shown in Fig 6 and 7. It is readily seen that within 22 hrs the distinction in the distribution of cRBC and aRBC is very marked and would thus constitute a valuable biological assay for sequestration of damaged RBC from circulation.

5. Dose Response

The experiment was repeated with different amounts of RBC. Figure 8 shows that a dose greater than 10^8 RBC cannot be effectively sequestered in 24 hr by a rat weighing 180g.

6. Specificity of Response

Glutaraldehyde treated RBC (GaRBC) (6) behave quite differently to cRBC and aRBC. GaRBC are retained in the peritoneal cavity, Table XIII. Further examination of the contents of the peritoneal cavity indicates that most of the GaRBC are taken up by the peritoneal macrophages, emphasizing the specificity of the biological response to the sialidase and glutaraldehyde treated RBC respectively.

7. Response to Heterologous RBC

It is obvious that the rat peritoneal assay is extremely sensitive, and a highly specific screening assay for erythrocytes and modified erythrocytes. In the experiments discussed so far, we have, unless otherwise stated, used a homologous system. Since the response obtained differs little from autologous system, one can only conclude that the rats used were from a highly inbred strain. When, however, heterologous RBC are used, the specificity of the system becomes even more evident. After 24 hrs, only about 20% of the radioactively labeled RBC can be accounted for in the liver, blood, kidney, spleen and peritoneal cavity. Focussing attention on the liver as the primary organ for removal of enzyme treated RBC, we obtain the pattern of results shown in Table XIV, 24 hr after i.p. injection of RBC.

ABBREVIATIONS

A_5	Acidic pentasaccharide alditol
A^+H^+-GM	A commercial pool of A^+H^+ hog gastric mucins
a-OSM	Asialo-ovine submaxillary mucin
A^+PSM	A-active porcine submaxillary mucin
aRBC	Asialo-erythrocytes
A-zyme	(α1→3) N-acetyl-D-galactosaminidase, enzyme that destroys A activity
β-Galase	β-galactosidase
β-GlcNAcase	β-N-acetylglucosaminidase
B-zyme	(α1→3) D-galactosidase, enzyme that destroys B activity
cRBC	control eyrthrocytes
DTT	Dithiolthreitol
EDTA	Ethylene diamine tetra-acetate
Fuc	L-fucose
Gal	D-Galactose
GalNAc	N-acetyl-D-galactosamine
GaRBC	Glutaraldehyde treated RBC
HAT	Hypoxanthine, Aminopterine, Thymidine
H^+-PSM	H-active porcine submaxillary mucin
HT	Hypoxanthine, Thymidine
i.c.	Intracutaneous
i.p.	Intraperitoneal
i.v.	Intravenous
ManNGc	N-glycolylmannosamine
N_1	Neutral monosaccharide, N-acetylgalactosaminitol
N_4	Neutral tetrasaccharide alditol
NeuNGc	N-glycolylneuraminic acid
NPC	Non-parenchymal cells
OSM	Ovine submaxillary mucin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Parenchymal cells
R_e	Relative electrophoretic mobility
SDS	Sodium dodecyl sulfate
WBC	White blood cells

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Kogure T., Lowrie G. N. and Aminoff D.: (1979-80) The α-galactosidase (B-zyme) from Clostridium sporogenes (Maebashi). (In preparation)

LEGENDS TO FIGURES

Fig. 1. Polyacrylamide gel electrophoresis of A-zyme from step 6. Coomassie blue staining of gel compared to A-zyme activity, as determined on 1 mm slices.

Fig. 2. Release of GalNAc from A^+PSM. Details in text. A: additional substrate added at 4 hr, B: additional enzyme added at 4 hr, C: no additions.

Fig. 3. SDS-polyacrylamide gel electrophoresis of A-zyme. Coomassie blue staining pattern is shown above each panel. A: activity with, ●—● p-nitrophenyl-α-N-acetylgalactosaminide, X—X p-nitrophenyl-β-galactoside, O—O p-nitrophenyl-β-N-acetylglucosaminide. B: activity with ●—● A^+PSM, O—O a-OSM, X—X A^+H^+GM. C: activity with O—O neutral tetrasaccharide alditol and X—X acidic pentasaccharide alditol from A^+PSM. D: activity with O—O sheep Forsmann and O—O anti-A titer of human erythrocytes.

Fig. 4. Polacrylamide gel electrophoresis of B-zyme, at various steps of purification; stained for protein with Coomassie Blue and assayed for B-zyme activity on 1mm slices of an equivalent sample run in parallel.

Fig. 5. Substrate specificity of B-zyme. (See text for details.)

Fig. 6. Distribution of radioactively labeled homologous rat asialo-erythrocytes as a function of time after injection into the peritoneum.

Fig. 7. Distribution of radioactively labeled homologous untreated rat-erythrocytes as a function of time after injection into the peritoneum.

Fig. 8. Distribution of radioactively labeled homologous asialo-erythrocytes after 24 hrs. as a function of the number of RBC injected into the peritoneum.

TABLE I

PURIFICATION OF A-ZYME

Step	Volume ml	A-zyme u/ml	Sp. Act. u/mg	Yield %	Purif. factor	A-zyme Sialidase	A-zyme β-Galase	A-zyme β-GlcNAcase
1. Culture Filtrate	4000	2	0.1	100	1	0.1	0.1	0.04
2. 0-67% $AmSo_4$ ppt	79	69	0.5	76	4	0.1	0.1	0.05
3. Sephacryl S-200	243	18	3.8	61	29	0.1	0.1	0.08
4. DEAE-Sephacel	180	14	55	35	425	5.3	18	4.1
5. pH4.5 ppt	8.5	290	149	34	1175	9.0	29	47
6. RBC-0 Adsorption of impurities	2.5	500	1043	17	8025	542	53	79
Fold Purification with ref. to contaminant						4516	883	1975

TABLE II

RELATIVE ACTIVITY OF PURIFIED ENZYME

WITH p-NITRO-PHENYL GLYCOSIDES

p-nitro-phenyl glycoside	% relative Activity
α-D-GalNAc	100
β-D-GalNAc	0
α-D-GlcNAc	0
β-D-GlcNAc	1.2
α-D-Gal	0
β-D-Gal	1.9
α-L-Fuc	0

Table III

EFFECT OF ADDITION TO INCUBATION MIXTURE

Addition to Incubation Mixture	Final Concentration mM	Relative Activity %
no addition	---	100
Ca^{++}	1	87
Ca^{++}	10	63
Mg^{++}	1	90
Mg^{++}	10	85
Mn^{++}	1	78
Mn^{++}	10	58
Hg^{++}	1	10
Hg^{++}	10	2
EDTA	1	78
EDTA	10	82
DTT	10	98

TABLE IV

PRODUCTS OF A-ZYME WITH VARIOUS MUCINS

Substrate	Enzyme from Step 3		Enzyme from Step 6	
	1hr	24 hr	1 hr	24 hr
a-OSM	GalNAc	GalNAc	GalNAc	GalNAc
A^+PSM	GalNAc, Fuc Gal, NeuNGc (ManNGc)	GalNAc, Fuc, Gal NeuNGc, ManNGc	GalNAc	GalNAc
H^+PSM	Fuc, Gal, GalNAc NeuNGc, ManNGc	Fuc, Gal, GalNAc, NeuNGc, ManNGc	---	(GalNAc)
$A^+H,^+$ GM	GalNAc, GlcNAc, Fuc, Gal	GalNAc, GlcNAc Fuc, Gal	GalNAc	GalNAc (GlcNAc)

( ) Indicates trace amount

TABLE V

OLIGOSACCHARIDE COMPOSITION OF A PREPARATION OF A^+PSM

	Oligosaccharide	μmoles/lg mucin Oligosacch.	GalNAc
A_5	-O-GalNAc-Gal-GalNAc | | NeuNGc Fuc	23	46
A_4	-O-GalNAc-Gal | | NeuNGc Fuc	35	35
A_3	-O-GalNAc-Gal | NeuNGc	2	2
A_2	-O-GalNAc | NeuNGc	23	23
N_4	-O-GalNAc-Gal-GalNAc | Fuc	96	192
N_3	-O-GalNAc-Gal | Fuc	162	162
N_2	-O-GalNAc-Gal	22	22
N_1	-O-GalNAc	71	71
	Total	434	553

TABLE VI

SPECIFICITY OF THE TWO α-N-ACETYLGALACTOSAMINIDASES

SEPARATED ON PAGE - SDS WITH R_e=0.28 AND 0.40

	ENZYME	
	Re = 0.28	Re = 0.40
p Nitro-phenyl-α-N-acetylgalactosaminide	+	+
N_4, GalNAcl$\overset{\alpha}{\rightarrow}$3Gal → GalNAcol Fuc	-	+
A_5, GalNAcl$\overset{\alpha}{\rightarrow}$3Gal → GalNAcol Fuc NeuNGc	-	+
Forssman antigen, GalNAcl$\overset{\alpha}{\rightarrow}$3 GalNAc -	-	+
OSM, containing NAN 2$\overset{\alpha}{\rightarrow}$6GalNAc$\overset{\alpha}{\rightarrow}$Ser	-	-
aOSM, containing GalNAc$\overset{\alpha}{\rightarrow}$Ser	+	±
H^+PSM, containing no A^+ determinants	-	-
A^+PSM, see Table V for structures	-	+
A^++H^+, GM containing A^+ determinants	-	+
A^+ Erythrocytes	-	+

TABLE VII

KINETIC CONSTANTS FOR A-ZYME WITH VARIOUS SUBSTRATES

Subststrate	Km (mM)	Vmax (nmole/min)
p-nitro-phenyl-α-GalNAc	0.77	10.6
a-OSM	0.44	2.9
A^{+}PSM	0.93	0.73
GalNAc→Gal→GalNAcol | Fuc	4.2	7.1
GalNAc→Gal→GalNAcol | | Fuc NeuNGc	3.1	7.1

Table VIII

EFFECT OF A-ZYME ON ERYTHROCYTE AGGLUTINATION TITER

RBC	Anti-Serum	Control	Enzyme Treated
A	Anti-A	1:256	1:16
	Anti-H (Lotus)	0	1:16
B	Anti-B	1:64	1:64
	Anti-H (Lotus)	0	0
0	Anti-A	0	0
	Anti-H (Lotus)	1:4	1:4

TABLE IX

PURIFICATION OF B-ZYME FROM CL. SPOROGENES

Step	Volume ml	Activity u/ml	Specific Activity	% Yield	Purification Factor
1. Culture filtrate	9750	0.0026	0.003	--	
2. Millipore Concentrate	200	0.19	0.0066	100%	1
3. MeOH fraction 31-47%	10	1.45	0.069	64	11
4. Am.SO_4 Fraction 50-60%	1	5.14	2.64	23	400
5. Sephacryl S-200	10	0.46	4.18	21	633
6a. DEAE Sephacel	4.2	0.67	10.0	13	1515
6b. Peak Tube from Step 6a	0.7	1.23	16.60	4	2518

TABLE X

DISTRIBUTION OF RADIOACTIVITY

FOLLOWING I.P. INJECTION OF ^{51}Cr - LABELED RBC

WHOLE ANIMAL

	cRBC	aRBC
Liver	4.4	62.3
Blood	89.8	33.9
Peritoneum Suspension	1.4	.6
Spleen	2.2	1.2
Kidneys	1.3	.8
Lung	.8	1.2

WITHIN PERITONEUM

	cRBC	aRBC
Peritoneum Suspension	100	100
RBC	83	75
Macrophages	3.6	5.2

TABLE XI

DYNAMICS OF ASSAY

Blood Constituents	Intra-periton. aRBC, 24 hr.	Intra-veneous aRBC, 48 hr.	Intra-cutaneous aRBC, 48 hr.	Intra-peritoneal cRBC, 48 hr.
% of injected dose that appears in blood	17	14	.03	60
		% Distribution in Blood		
Whole Blood	100	100	100	100
Plasma	2.1	1.8	44.3	0.15
WBC, Total	0.02	0.03	0.06	0
RBC, (Calculated)	98	98	56	99

Rats were injected i.p., i.v., or i.c. with homologous aRBC or cRBC labeled with ^{51}Cr. Radioactivity in the blood constituents, separated by Percoll, were measured 24 hr. and 48 hr. later. Results are expressed as a percentage of that found in whole blood.

TABLE XII

ANATOMICAL DISTRIBUTION OF RADIOACTIVELY LABELED RBC

24 HR. FOLLOWING I.P. INJECTION OF aRBC AND cRBC

Organ	Component	aRBC cpm/10^6 RBC	cRBC
Liver[1]	NPC	695	N.D.[3]
	PC	161	N.D.
		% Distribution	
Peritoneal Cavity[2]	Supernatant	2.0	N.D.
	Macrophage	2.6	N.D.
	Granulocytes	0.5	N.D.
	RBC	94.9	N.D.
Blood[2]	Plasma	2.1	0.2
	WBC	0	0
	RBC	97.6	100

1) Liver cells separated by collagenase perfusion of liver and differential centrifugation of liver cells.

2) Peritoneal washing and whole blood were separated on Percoll.

3) N.D. not determined

TABLE XIII

ANATOMICAL DISTRIBUTION OF UNTREATED, cRBC, AND MODIFIED RBC, aRBC AND GaRBC, IN BLOOD, LIVER AND PERITONEUM

	% Distribution		
	cRBC	aRBC	GaRBC
Blood	90	9	6
Liver	8.5	90	16
Peritoneum	1.5	1	78

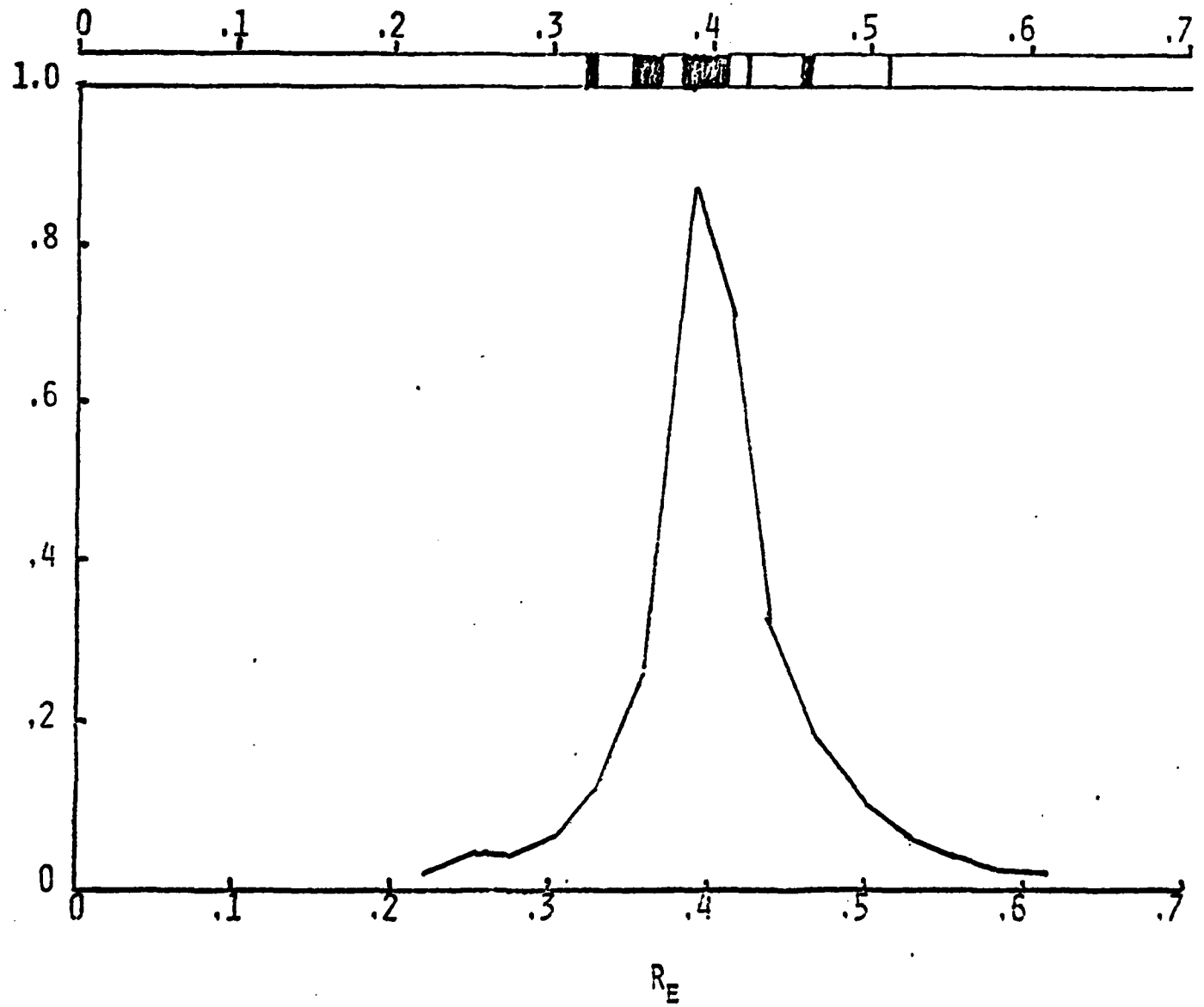
TABLE XIV

PERCENT OF INJECTED DOSE RECOVERED IN LIVER

24 HOURS FOLLOWING i.p. INJECTION

Type of Erythrocyte	Treatment	% in Rat Liver
Homologous		
Rat	None	5
Rat	Sialidase	80
Rat	Glutaraldehyde	3
Rat	N-ethylmaleimide	31
Heterologous		
Rabbit	None	13
Human A	None	7
Human B	None	19
Human O	None	8

FIG 1



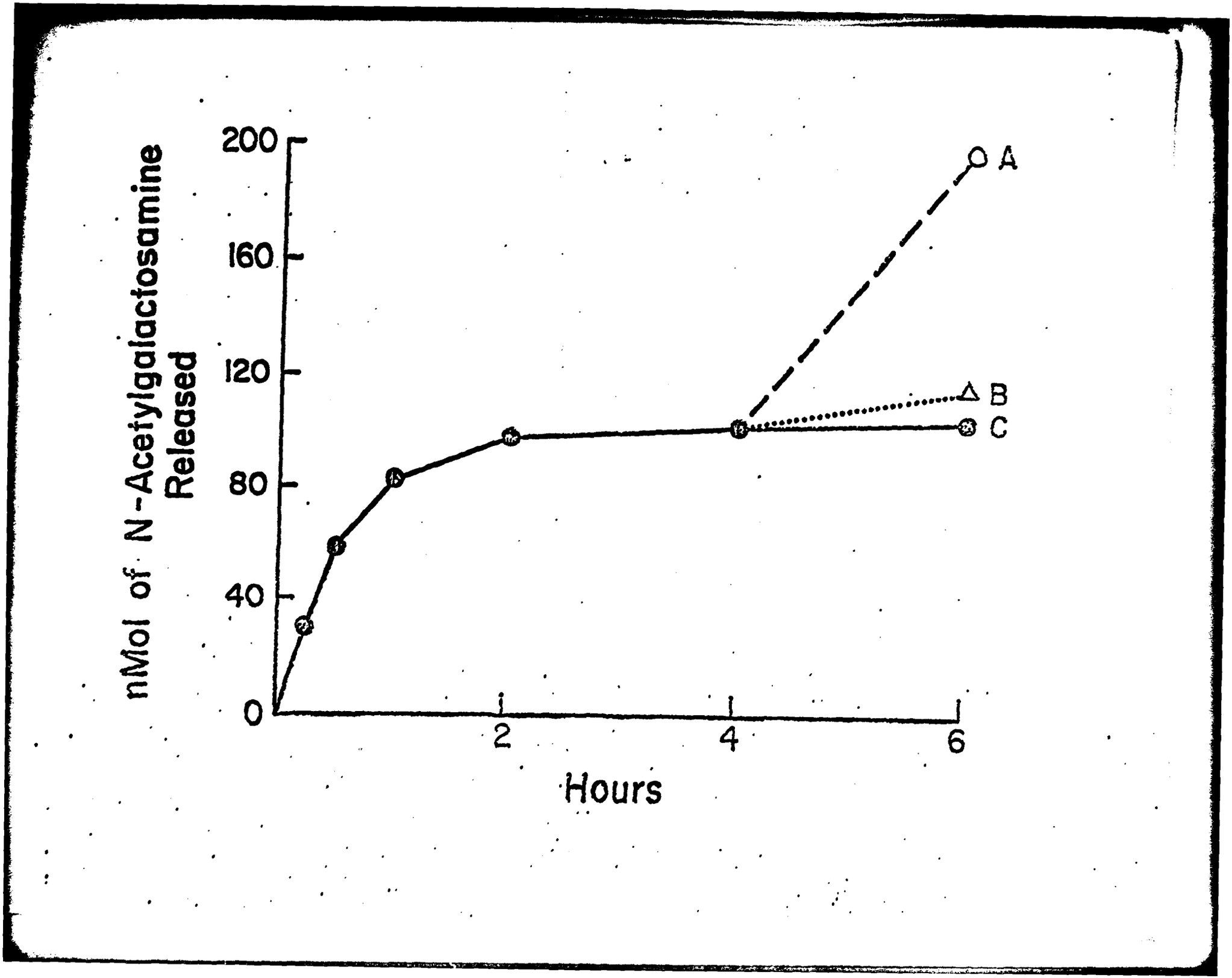
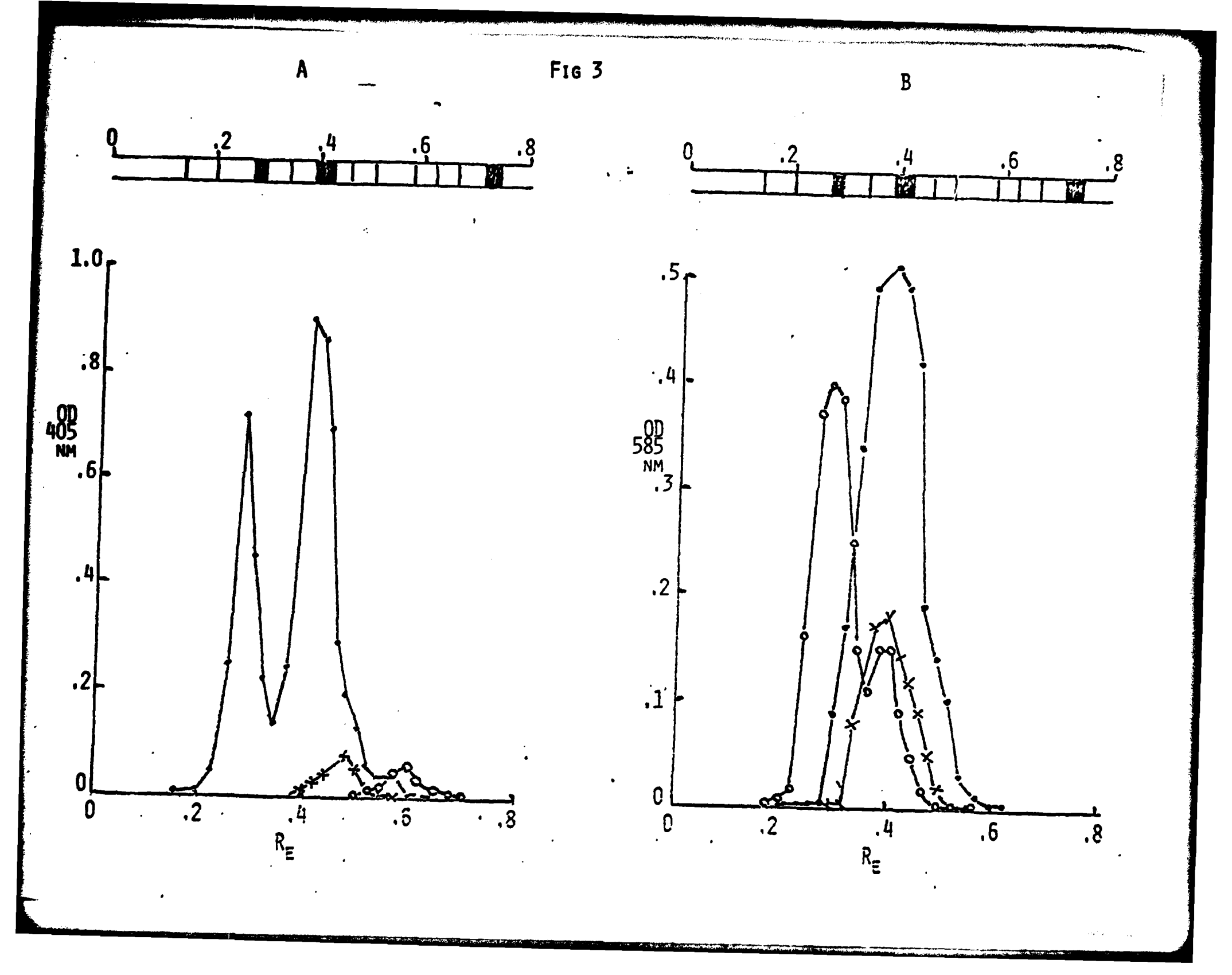

nMol of N-Acetylgalactosamine Released
200
160
120
80
40
0
2
4
6
Hours
A
B
C

FIG 3



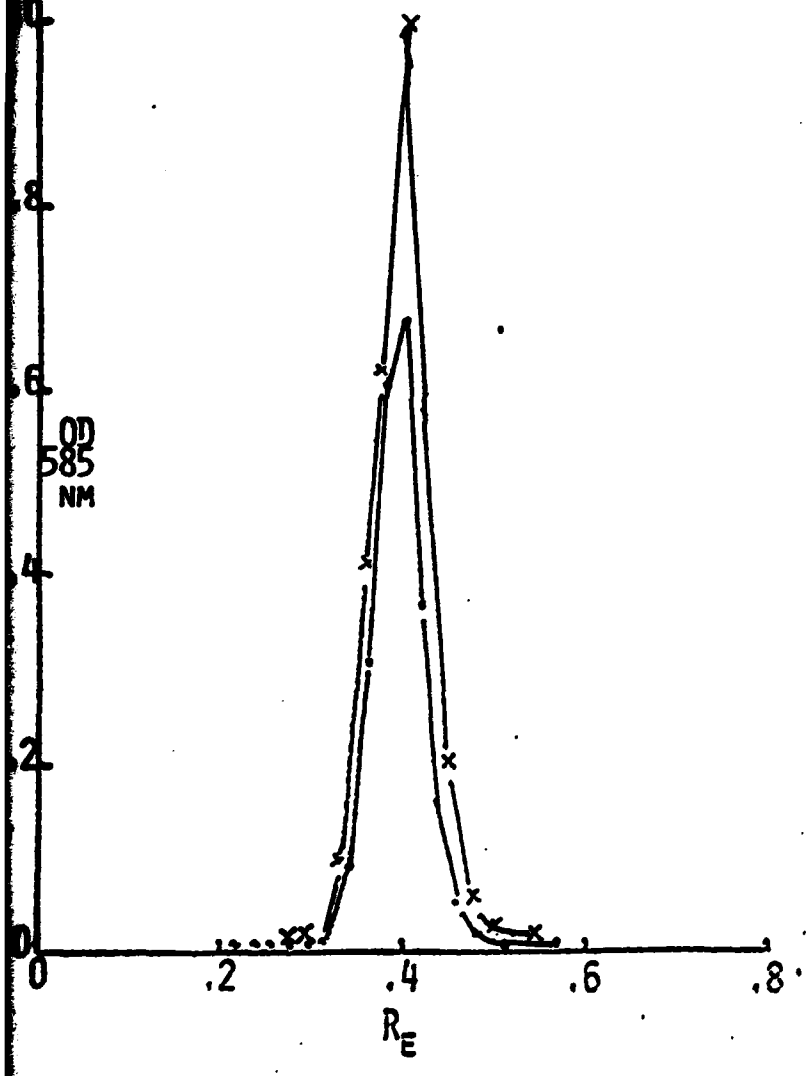
C

FIG 3

D


0
.2
.4
.6
.8


0
.2
.4
.6
.8


OD
585
NM
0
.2
.4
.6
.8
R_E

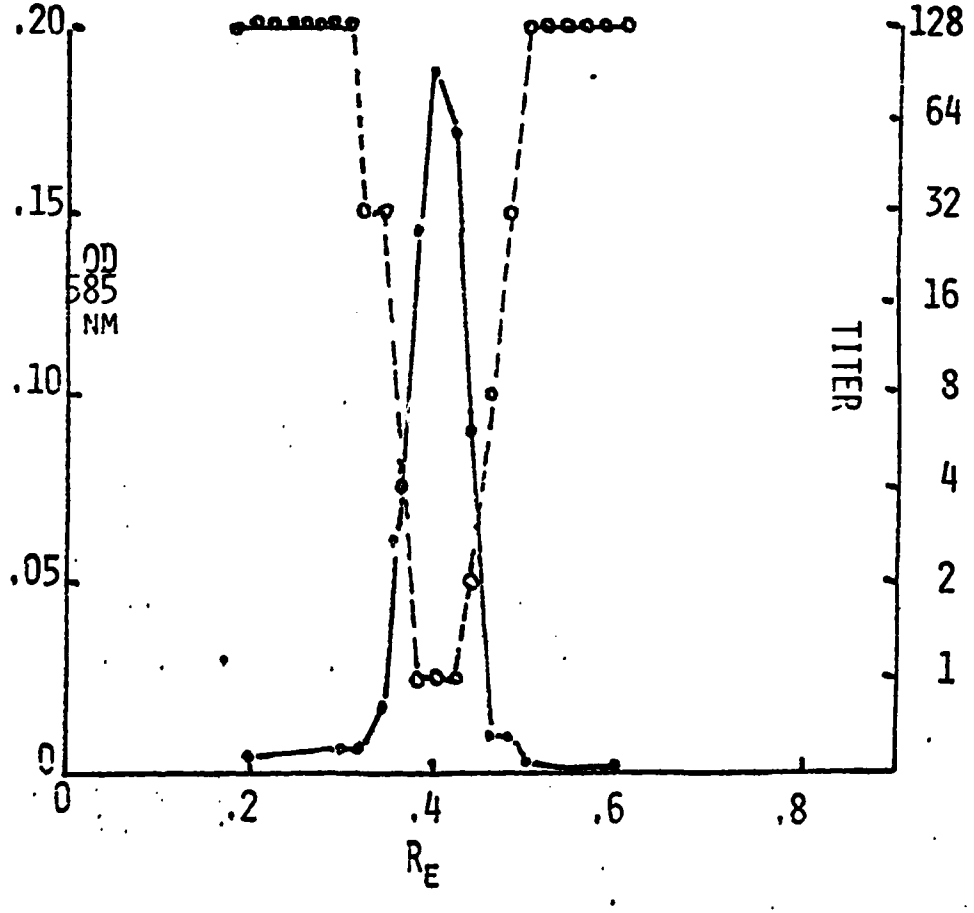

.20
.15
.10
.05
0
OD
585
NM
0
.2
.4
.6
.8
R_E
128
64
32
16
8
4
2
1
TITER

FIG 4

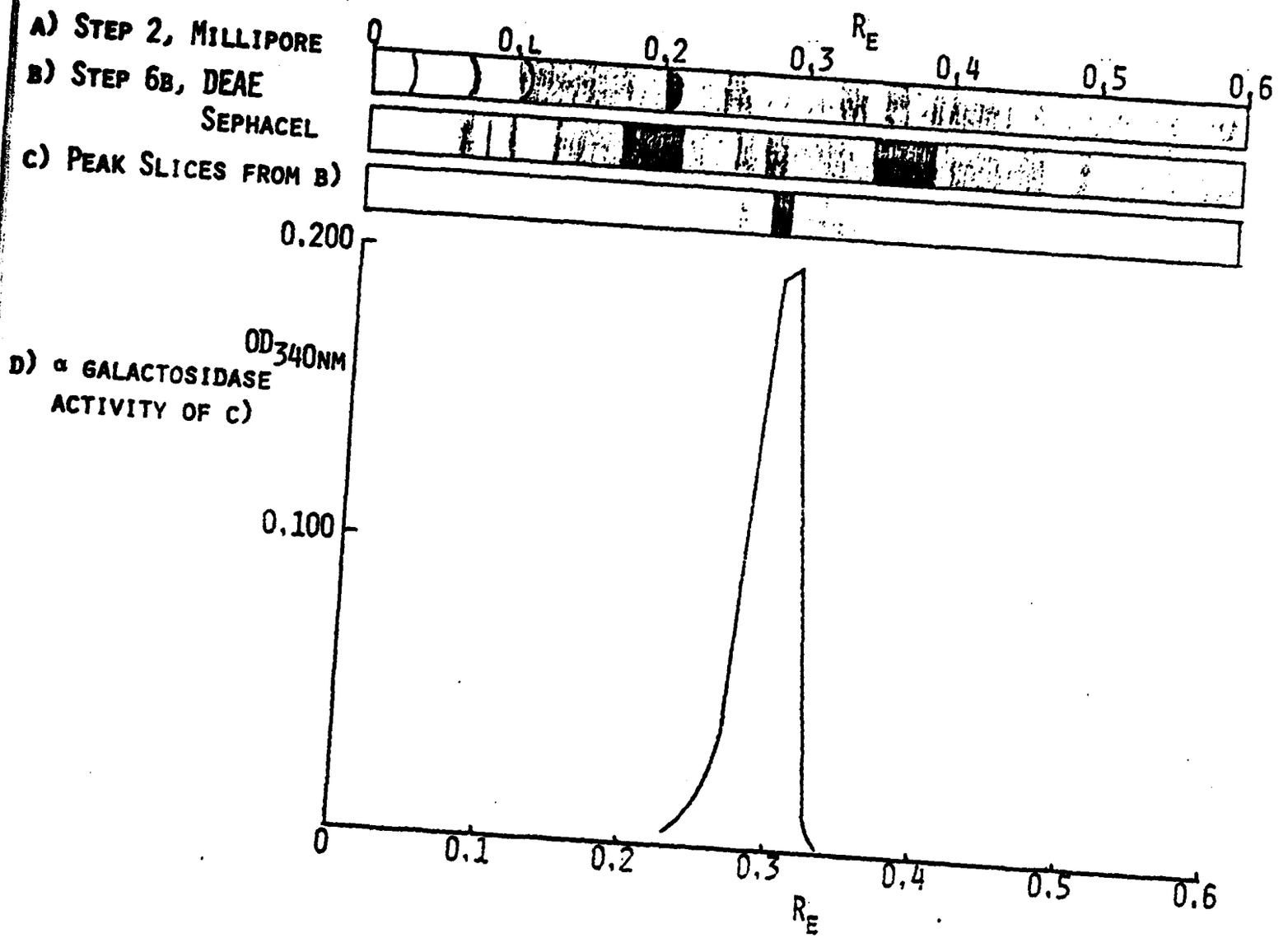

A) STEP 2, MILLIPORE
B) STEP 6B, DEAE SEPHACEL
C) PEAK SLICES FROM B)
D) α GALACTOSIDASE ACTIVITY OF C)
0
0,1
0,2
0,3
0,4
0,5
0,6
R_E
0.200
0.100
OD_{340NM}

FIG 5

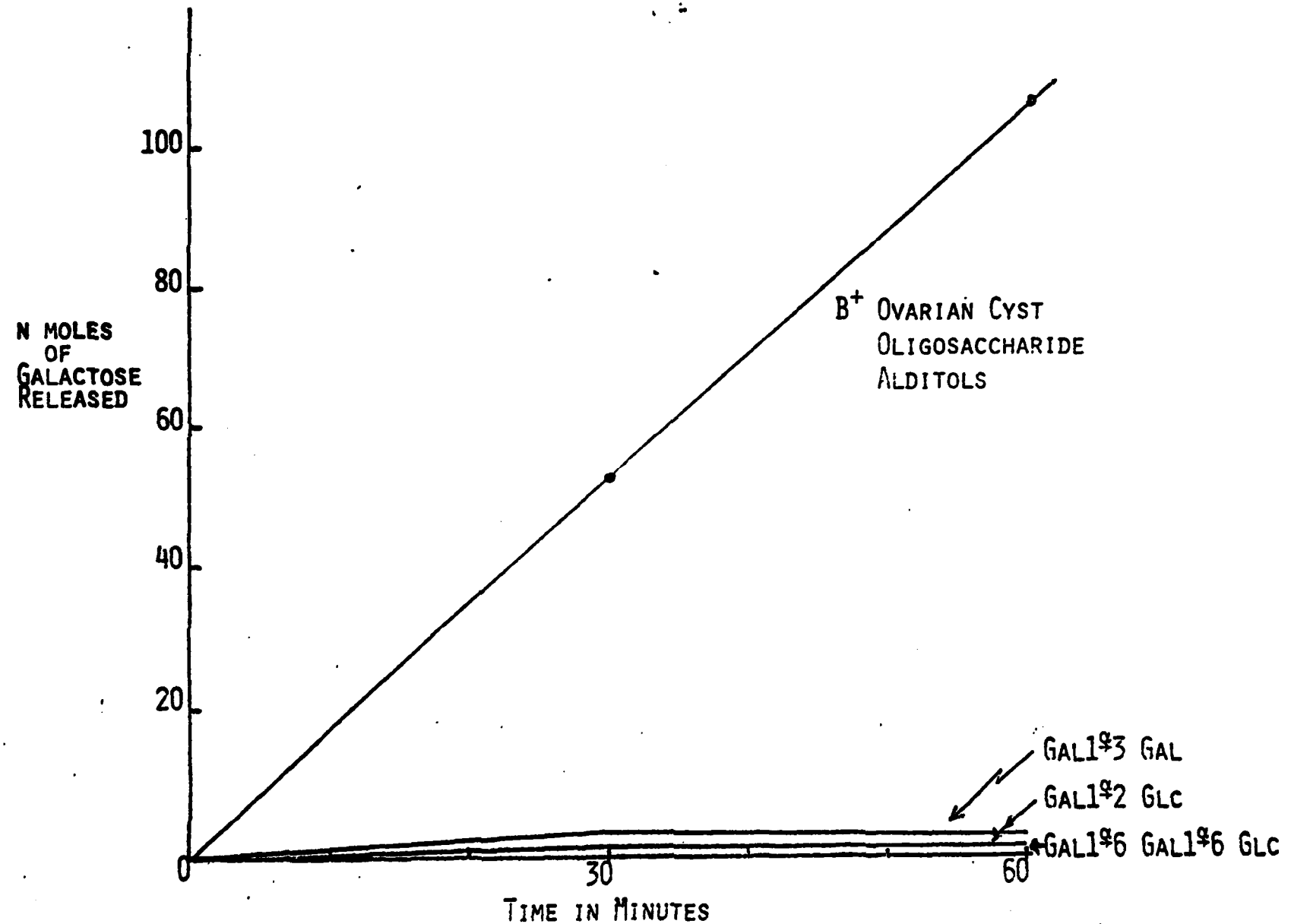

N MOLES OF GALACTOSE RELEASED
100
80
60
40
20
0
30
60
TIME IN MINUTES
B+ OVARIAN CYST OLIGOSACCHARIDE ALDITOLS
GAL1→3 GAL
GAL1→2 GLC
GAL1→6 GAL1→6 GLC

FIG 6

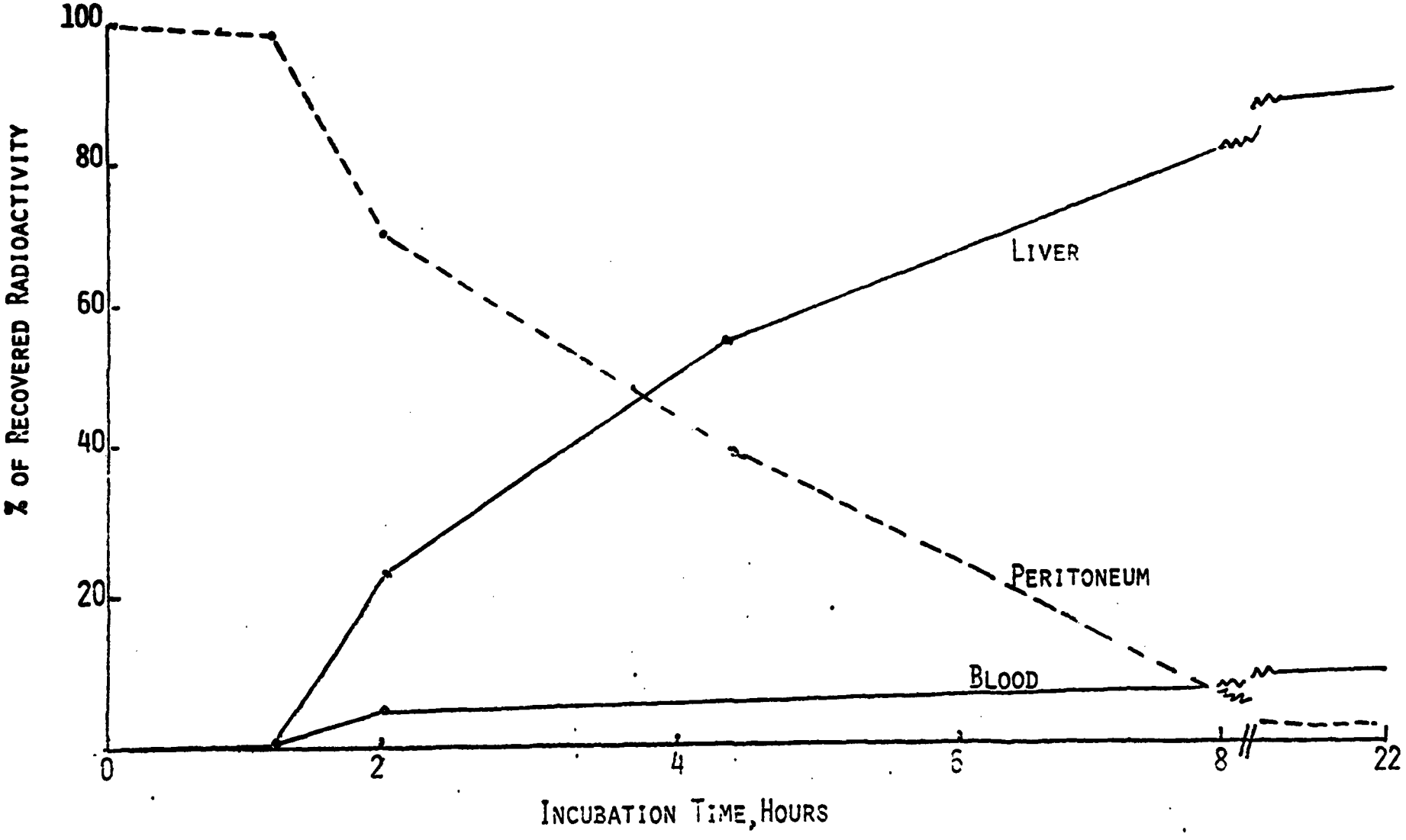

100
80
60
40
20
0
2
4
6
8
22
% OF RECOVERED RADIOACTIVITY
LIVER
PERITONEUM
BLOOD
INCUBATION TIME, HOURS

FIG 7

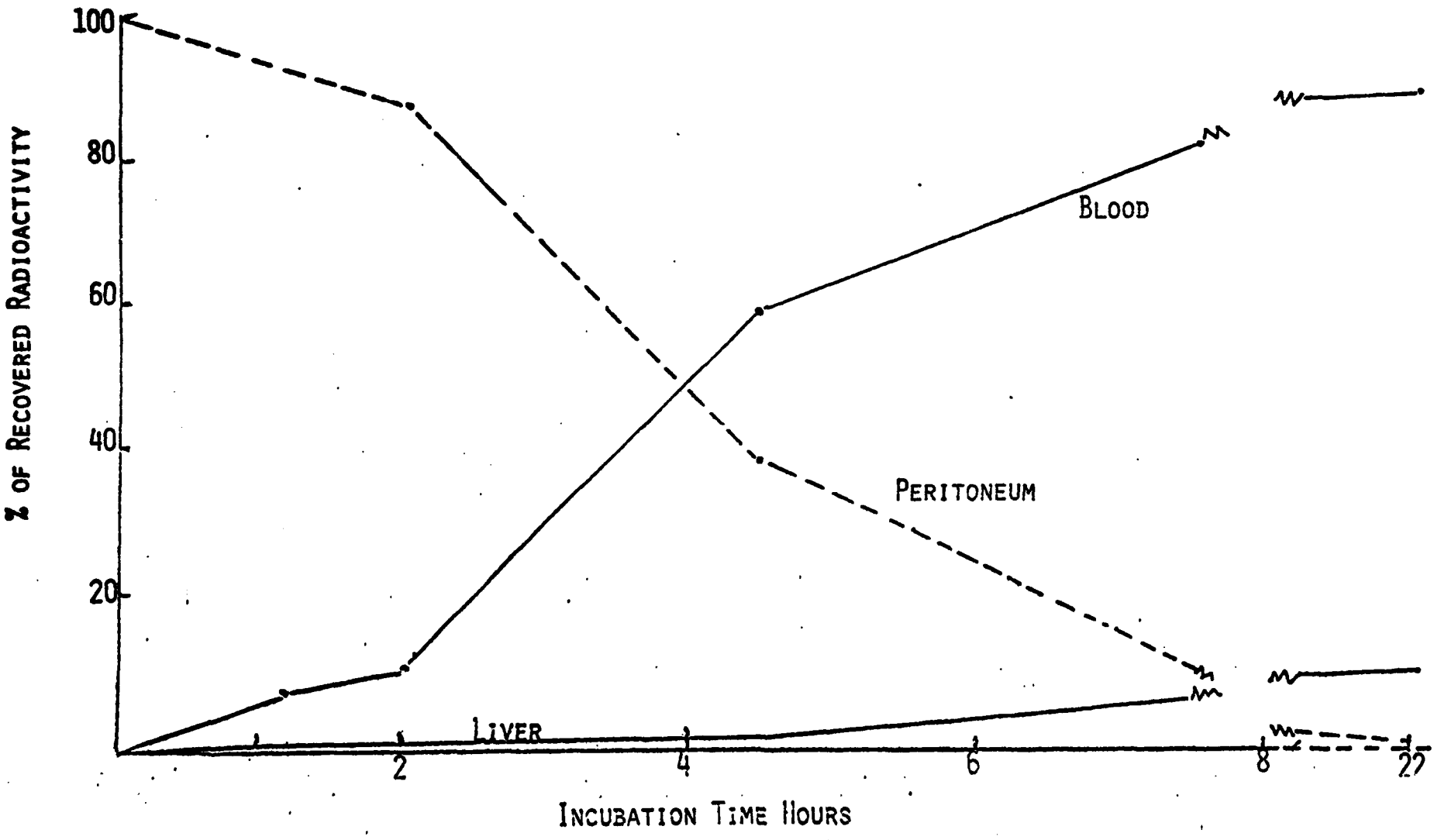
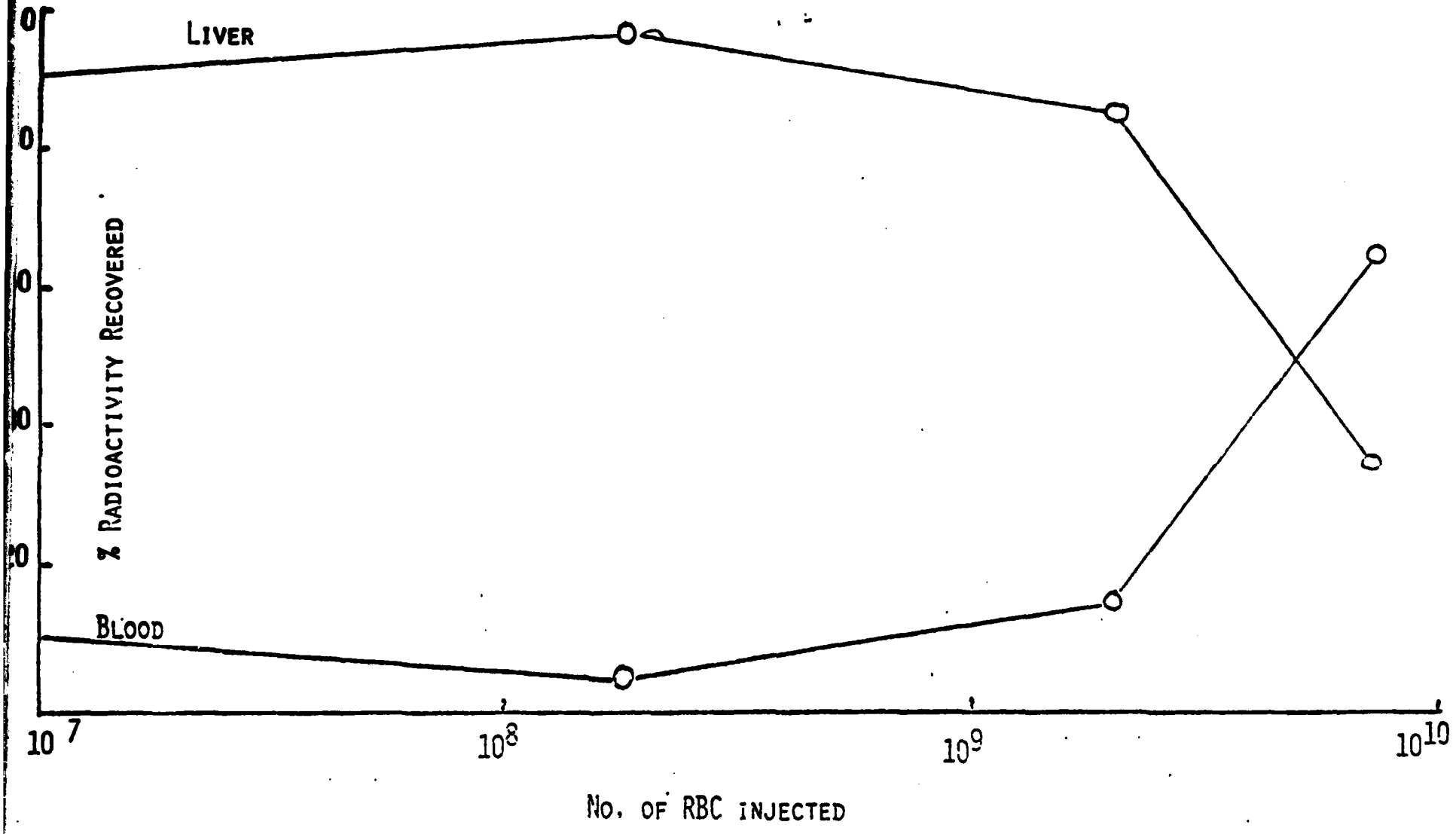

100
80
60
40
20
% OF RECOVERED RADIOACTIVITY
BLOOD
PERITONEUM
LIVER
2
4
6
8
27
INCUBATION TIME HOURS

FIG 8


LIVER
BLOOD
% RADIOACTIVITY RECOVERED
10^7
10^8
10^9
10^{10}
No. OF RBC INJECTED